REMARKS

I. Status of the Claims

Claims 63-104 and 108-112 are pending and under examination. Claims 1-62 and 105-107 were canceled previously, without prejudice or disclaimer. Applicants reserve the right to file one or more continuing applications to the canceled subject matter.

The Office highlighted several grammatical and typographical mistakes in the pending claim set. See "Claim Objections" at page 3. Applicants believe the presently-amended claim set addresses each of those points and therefore believe they have overcome those objections.

II. New Matter Objection under 35 U.S.C. § 132(a)

The Office maintains that Applicants' preliminary amendment of June 7, 2004, introduces new matter under 35 U.S.C § 132(a) because, it is alleged, the contents of the amendment ("Examples 17-31, everything relating to Figures 12-20, [and] every reference to monomer and exogenous factors") are "not supported by the original disclosure." Final Office action at page 3.

Applicants regret that the "new matter" issue is not yet rectified. To allay any confusion at Examiner Kaufman's end, they attempt in good faith to explain why the subject matter identified by the examiner as "new" in fact does ***not*** violate Section 132(a). Briefly, Applicants' papers dated November 18, 2003, which apparently were mishandled somewhere within the PTO, nevertheless were timely filed and were recorded by the Office on November 28, 2003. The sequence of relevant events, to Applicants' knowledge, is as follows:

1. Applicants deposited the specification text with the PTO on November 26, 2003.

2. Two days later, on November 28th, Applicants filed (i) a new substitute specification, and (ii) an inventor declaration requesting a priority date of November 28, 2003, for the then-completed application, as opposed to the earlier date of November 26th.

3. The November 28th filing was a submission of the following documents:

(i) Preliminary Amendment (4 pages);

(ii) Transmittal of Formal Drawings (1 page);

(iii) Formal Drawings (34 sheets, Figures 1-4, 5a-5f, 6-10, 11a-11b, 12a-12b, 13, 14a-14b, 15a-15b, 16a-16c, 17a-17f, 18a-18b, 19a-19b, and 20);

(iv) Substitute Sequence Listing (20 pages);

(v) Marked-up Substitute Specification (145 pages); and

(vi) Clean Substitute Specification (145 pages).

Applicants received a PTO postcard, date-stamped November 28, 2003, which confirmed receipt of all of these documents.

4. For reasons that remain unknown to Applicants, the PTO never apparently logged or uploaded the actual November 28, 2003, filing to their internal docketing system or to PAIR, despite Applicants' successful deposit of those materials, as evidenced by the return by the PTO of a date-stamped postcard indicating the November 28th submission.

5. Examiner Kaufman, therefore, was aware only of the incomplete specification deposit date of November 26, 2003, as evidenced by her statement that, "while the inventor declaration says a preliminary amendment and substitute specification was filed 11/28/2003, nothing is of record as being received on that day . . . the records . . . show only original submissions on 11/26/03, with the next submission [on] 6/7/04." Final Office action at page 4.

6. But the "6/7/04"-dated "submission" appears to be Applicants' true and original November 28th filing. The PAIR website's "Image File Wrapper" tab for this matter, USSN 10/721,763, might indicate a PTO mail room date of "6/7/04," but each of the hyperlinked documents associated with those 6/7/04 entries is signed and dated "November 28, 2003," *not* June 7, 2004. Accordingly, it appears that the PTO finally uploaded

Applicants November 28th filing but inadvertently date-stamped its pages with the wrong date before uploading to PAIR.

7. In a conversation with Examiner Kaufman on January 18, 2008, Applicants' representative related these events and, in accordance with her request, provided the examiner with copies of all of the document of November 28, 2003, totaling 353 pages, as indicated in paragraph 3 above, as well as with a copy of the PTO's "date-stamped postcard," showing receipt of these documents by the PTO. Please see the "Miscellaneous Incoming Letter" dated January 22, 2008, on the USPTO PAIR website "Image File Wrapper" tab for this matter, USSN 10/721,763.

Accordingly, the preliminary amendment in question **did not introduce new matter because it was filed concurrently with a new substitute specification on November 28, 2003**. This comports with Applicants' inventor declaration, also concurrently filed at that time, which requested a priority date of November 28, 2003, for the *completed* application, as opposed to the earlier date of November 26th, when the specification text was *deposited* with the PTO. In other words, the subject matter of the preliminary amendment is not "new" with respect to documentation on file by November 28th. Accordingly, Applicants have not canceled the matter which the Office purports to be "new."

Applicants sincerely hope that their efforts to rectify an inadvertent PTO mailroom error will highlight why there is no issue of "new matter" under Section 132(a) and why the rejections under 35 U.S.C. §§ 132(a) and 112, first paragraph (pages 3 and 4 of the Final Office action), therefore are moot.

III. Rejections under 35 U.S.C. 112, second paragraph

Claims 64, 69, 74, 79, 84, 89, and 109-112 and their dependent claims are rejected under 35 U.S.C. § 112, second paragraph as allegedly indefinite.

With respect to claims 64, 69, 74, 89, and 109-112, Applicants have amended step (2) recited in those claims to clarify that the antibody, or its functional fragment, "*binds to* TRAIL-R2" instead of asserting that it is "*bound to*" TRAIL-R2. The original recitation was an unfortunate and unintended consequence of Japanese-to-English translations and communication between Applicants and their representative; and, furthermore, this

amendment comports with Examiner Kaufman's suggestion at page 6 ("Replacement of 'bound to' with "which binds to' would obviate this rejection").

Step (4) of these claims also has been amended to clarify that survival measurement steps entail measuring the absorbance of wells in the 96-well flat-bottomed plate that are carcinoma cell-free, as well as those that are control wells containing carcinoma cells and a control antibody.

Claims 84 and 89 are amended to clarify the antecedent basis for "carcinoma cells" by adopting the Examiner's suggestion to place "said" before "containing" (page 6 of the Office Action).

Claim 108 is amended to comport with the Examiner's suggestion to "replac[e] 'a fragment thereof' with –an extracellular fragment thereof--'.

Claim 111 is amended to clarify what is the antibody used in the recited test, i.e., it is the antibody of claim 108, from which claim 111 depends.

None of these amendments introduce new matter and therefore Applicants respectfully request their entry into the record.

IV. Claim Interpretation

Examiner Kaufman correctly interprets "antibody monomer" to be an antibody that is a "monomer [that] is not crosslinked or bound to another substance, including another antibody." Office Action at page 2. She also correctly asserts that "an antibody polymer is an antibody which is necessarily crosslinked to another substance, such as a secondary antibody." Id.

What is important to note, however, is that the antibody polymer forms a complex with a number of the same antibody molecules. By contrast, the monomeric antibody is not so crosslinked. Applicants state in their specification that "[A]n antibody which is not the 'monomer of an antibody' or 'monomer antibody' includes an antibody in which plural antibodies form a complex, and is said as a 'polymer (multimer) of antibodies' or 'polymer (mulitmer) antibody'. The complex formed by plural antibodies can be formed by the cross-

linker mentioned above or by the polymerization of two or more of antibody molecules" (see paragraph [0077] of US2005/0249729).

For the reasons set forth below, Applicants submit that none of the prior-art antibodies is monomeric. That is, the cited prior art antibodies are only active when they are aggregated together in a polymeric complex.

V. Declaration Evidence

Applicants submit herewith a presently-unexecuted declaration prepared by co-inventor, Dr. Kazuhiro Motoki, Senior Scientist at KIRIN PHARMA COMPANY LTD., who relates the results of experiments that he and his team conducted on the cited prior art antibodies. Applicants are waiting to receive an identical copy of the declaration signed and dated by Dr. Motoki at the time this present paper is being filed; and will forward that version to the Patent Office once the undersigned has received it.

In his declaration, Dr. Motoki concludes that none of the prior art documents discloses a *monoclonal* antibody, or a functional fragment thereof, that binds to TRAIL-R2 and thereby induces apoptosis in carcinoma cells. From Dr. Motoki's analysis, it was apparent that only when the prior art antibodies are aggregated into a polymeric complex do they exhibit apoptotic activity. Accordingly, in the words of the claimed invention, a prior art antibody is ***not*** a "single substance without forming a polymer [that] binds to TRAIL-R2 and induces apoptosis in carcinoma cells expressing TRAIL-R2, independently of exogenous factors other than the antibody and the functional fragment thereof."

(i) 16E2

To elaborate, Examiner Kaufman states that the 16E2 prior art antibody induces apoptosis in carcinoma cells (SK-MES-1) when not cross-linked.

The 16E2 antibody is a single-chain Fv (scFV) as described in U.S. Patent No. 6,342,369 (col. 11, lines 41 to 55). Generally, scFV forms a polymer without cross-linking. See Kortt *et al.*, *Protein Engineering* 10: 423, 1997 (appended), which demonstrates that scFV forms a polymer. Specifically, Kortt showed that dimers and trimers of scFV antibodies were formed after gel filtration and not only individual, unaggregated antibodies.

Please see Figures 1 and 5 of Kortt. Accordingly, it is very likely that Griffith's preparation of 16E2 antibodies would naturally contain polymeric forms of the antibody.

Applicants proved that this is indeed the case: they produced the 16E2 antibody according to the method disclosed in U.S. Patent No. 6,342,369 and examined whether or not the antibody form a polymer. Exhibit A to the declaration shows that almost all of the 16E2 antibodies formed dimeric and tetrameric polymers. Applicants detected hardly any monomeric 16E2. It is beyond question, therefore, that the apoptosis-inducing effects of Griffith's 16E2 antibody is attributable to its polymeric composition. 16E2 does not function as "a single substance without polymer."

(ii) TRA-8

Examiner Kaufman also says that the TRA-8 antibody induces apoptosis in carcinomic Jurkat leukemia cells, when not cross-linked. Example 8 of US2003/0190687 allegedly shows that the TRA-8 antibody has apoptosis-inducing activity.

This proved to be untrue: Applicants tested that TRA-8 antibody preparation and found that the monomeric TRA-8 antibody does not induce apoptosis. See Exhibit B appended to the Declaration. The TRA-8 antibody had no apoptotic activity when it existed in culture medium but was able to induce apoptosis only when it was cross-linked (Experiment 1 of Exhibit B). Applicants demonstrated that the purified TRA-8 antibody contains both antibody monomer and polymer (see Experiment 2 of Exhibit B), and that the purified TRA-8 antibody had the activity of inducing apoptosis without any cross-linker (see Experiment 3 of Exhibit B). The antibody monomer was isolated from the purified TRA-8 antibody (Experiment 4) and the antibody monomer did not have the activity of inducing apoptosis (Experiment 5). Therefore, the monomer of the TRA-8 antibody does not exhibit apoptosis-inducing activity. Only the polymeric form of TRA-8 has apoptosis-inducing activity.

(iii) M413

Examiner Kaufman states that Griffith's M413 antibody induces apoptosis in carcinoma cells (WM9) when it is not cross-linked, *i.e.*, when it is not polymeric. Generally, however, antibodies ***do*** form an aggregate of the same antibody molecules even without

cross-linking. For instance, Applicants demonstrated that a preparation of the purified antibody of Example 21 of the application contained ***both*** monomeric and polymeric forms of the antibody. See Example 28, which further showed that the monomeric "0304" and "0322" antibodies exhibited cell-death-inducing activity on Colo205 cells, but the monomeric "H-48-2" antibody had no cell-death inducing activity on Colo205 cells. That is, the H-48-2 antibody only exhibited cell-death-inducing activity when it was in the form of polymer. Please see paragraph [0404] of the published application US2005/0249729.

Accordingly, it is possible that the supposed apoptotic effect reported by Griffith concerning the M413 antibody is attributable to a naturally-aggregated polymeric fraction of the M413 antibody preparation, and not to a 100% preparation of a monomeric form of the antibody. It is important to note that the true properties of the M413 antibody cannot be confirmed or tested because it is not available to the public: the M413 antibody is not available commercially and it has not been deposited in the depository institution.

VI. Griffith does not anticipate because it does not teach a monomeric agent that itself induces apoptosis via binding to TRAIL-R2

Claims 63-104 and 108 remain rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Griffith *et al.*, *J. Immunol.* 162: 2597, 1999 ("Griffith"). According to the Office, Griffith raised several monoclonal antibodies to various TRAIL receptors and tested them for apoptosis-inducing activity. Office action at page 7.

For the reasons discussed in subsection V above and elaborated upon in the accompanying Declaration, Applicants assert that Griffith does not teach a "monoclonal antibody or a functional fragment thereof, which is a single substance without forming a polymer, binding to TRAIL-R2 and induces apoptosis in carcinoma cells expressing TRAIL-R2, independently of exogenous factors other than the antibody and the functional fragment thereof." Griffith's antibodies only induce apoptosis when they are *polymeric* form. Griffith does not therefore anticipate the claimed invention which explicitly requires apoptosis to be induced by a monomeric agent, and expressly disclaims the polymeric form ("a single substance without forming a polymer"). Griffith does not anticipate the claimed invention and therefore Applicants respectfully request withdrawal of this rejection.

V. Neither U.S. Patent No. 6,342,369 nor U.S. 2003/0190687 teaches production of monoclonal antibodies that induce, without polymerization of such antibodies, the death of cells that express TRAIL-R2

(i) U.S. Patent No. 6,342,369 (antibody 16E2)

Claims 63-104 and 108 are rejected under 35 U.S.C. § 102(e) as allegedly anticipated by United States Patent 6,342,369 (issued January 29, 2002). According to the Office, the '369 patent described methods that "did not use the same assay used to characterize the claimed antibody . . . but it appears absent evidence to the contrary that antibody 16E2 has the required functional properties required by the instant claims." Office action at page 8.

In fact, the '369 patent does *not* describe an antibody that is "a single substance" that binds to TRAIL-R2 receptors that are expressed in carcinoma cells and thereby induces death of those cancerous cells, as explained in subsection V and the accompanying Declaration. The '369 patent does not describe a preparation of active monomeric 16E2, but only active apoptosis-inducing properties attributable to the polymeric form of 16E2. Accordingly, the '369 patent does not anticipate the claimed invention which explicitly requires apoptosis to be induced by a monomeric agent, and expressly disclaims the polymeric form ("a single substance without forming a polymer"), and Applicants therefore respectfully request withdrawal of this rejection.

(ii) U.S. 2003/0190687 (antibody TRA-8)

Claims 63-104 and 108 are rejected under 35 U.S.C. § 102(e) or 102(a) as allegedly anticipated by United States application publication U.S. 2003/0190687 (published October 9, 2003; priority to May 2, 2001), now U.S. Patent No. 7,244,429. The antibody, TRA-8, "appears to have all the properties" of the claimed antibody.

As Applicants demonstrated in subsection V and in the accompanying declaration, TRA-8 cannot induce apoptosis "as a single substance without forming a polymer." Applicants proved that TRA-8 can only induce apoptosis when it forms a polymer by aggregation. Accordingly, the '429 patent does not anticipate the claimed invention which explicitly requires apoptosis to be induced by a monomeric agent, and expressly disclaims

the polymeric form ("a single substance without forming a polymer"), and Applicants therefore respectfully request withdrawal of this rejection.

CONCLUSION

Applicants invite Examiner Kaufman to contact the undersigned directly, in the event that she believe any of the salient issues to warrant further consideration.

Respectfully submitted,

Date March 26, 2008 By V.S. Mohan

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The Commissioner is authorized to charge any additional fees, which may be required under 37 CFR §§ 1.16 - 1.17, and to credit any overpayment to Deposit Account No. 19-0741. Should no proper payment accompany this response, then the Commissioner is authorized to charge the unpaid amount to the same deposit account. If any extension is needed for timely acceptance of submitted papers, Applicants hereby petition for it under 37 CFR §1.136 and authorize payment of applicable fee(s) from the deposit account.

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Single-chain Fv fragments of anti-neuraminidase antibody NC10 containing five- and ten-residue linkers form dimers and with zero-residue linker a trimer

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Single-chain variable fragments (scFvs) of anti-neuraminidase antibody NC10 were constructed by joining the V_H and V_L domains with 10-residue $(Gly_4Ser)_2$ and five-residue (Gly_4Ser) linkers; a zero-residue linker scFv was constructed by joining the C-terminal residue of the V_H domain to the N-terminus of the V_L domain. The scFv with the 10- and five-residue linkers exclusively formed dimeric antibody fragments (M_r 52 000). These were shown to be bivalent and were able to cross-link two neuraminidase tetramers to form a 'sandwich' type complex; each antigen combining site could also bind an anti-idiotype Fab'. The zero-residue linker scFv (M_r 70 000) was shown to form a trimer with three active antigen combining sites, each binding an anti-idiotype Fab' to yield a complex of M_r 212 000. The orientation of the combining sites in the zero-residue linker scFv, however, was such that it could not cross-link tetramers of neuraminidase. BIAcore biosensor experiments showed that the affinity of each individual antigen combining site in both the 10- and five-residue linker scFv dimers and zero-residue linker scFv trimer was essentially the same when the scFvs were immobilized onto the sensor surface. However, when the scFvs were used as the analyte, the dimeric and trimeric scFvs showed an apparent increase in binding affinity due to the avidity of binding the multivalent scFvs.

Keywords: antibody/complexes with antigens/dimers/shorter linkers/single-chain Fvs/trimers

Introduction

Recombinant single-chain variable fragments (scFvs), in which the two variable domains are covalently joined via a flexible peptide linker have been shown to fold in the same conformation as the parent Fab (Kortt *et al.*, 1994; Zdanov *et al.*, 1994). ScFvs usually show the same binding specificity and affinity as the monomeric form of the parent antibody (Bedzyk *et al.*, 1990; Pantoliano *et al.*, 1991) and exhibit improved stability compared with Fv fragments, which are not associated by covalent bonds and may dissociate at low protein concentrations (Glockshuber *et al.*, 1990). ScFv fragments have been secreted as soluble, active proteins into the periplasmic space of *Escherichia coli* (Glockshuber *et al.*, 1990; Anand *et al.*, 1991). Furthermore, various protein linking strategies have been used to produce bivalent or bispecific scFvs as well as bifunctional scFv fusions and these reagents have numerous applications in clinical diagnosis and therapy. The linking strategies include the introduction of cysteine residues into an scFv monomer followed by disulphide linkage to join two scFvs (Cumber *et al.*, 1992; Adams *et al.*, 1993; Kipriyanov *et al.*, 1994; McCartney *et al.*, 1995). Linkage between an scFv pair can also be achieved via a third polypeptide linker (Gruber *et al.*, 1994; Mack *et al.*, 1995; Neri *et al.* 1995). Bispecific or bivalent scFv dimers have also been formed using the dimerization properties of the kappa light chain constant domain (McGregor *et al.*, 1994) and domains such as leucine zippers and four helix bundles (Pack and Pluckthun, 1992; Pack *et al.*, 1993, 1995; Mallender and Voss, 1994). Bifunctional scFv fusion proteins have been constructed by attaching molecular ligands such as peptide epitopes for diagnostic applications (Lilley *et al.*, 1994; Coia *et al.*, 1996), enzymes (Wels *et al.*, 1992; Ducancel *et al.*, 1993), streptavidin (Dubel *et al.*, 1995) or toxins (Chaudhary *et al.*, 1989, 1990; Batra *et al.*, 1992; Buchner *et al.*, 1992) for therapeutic applications.

In the design of scFvs, peptide linkers were engineered to bridge the 35 Å distance between the carboxy terminus of one domain and the amino terminus of the other domain without affecting the ability of the domains to fold and form an intact binding site (Bird *et al.*, 1988; Huston *et al.*, 1988). The length and composition of various linkers were investigated (Huston *et al.*, 1991) and linkers of 14–25 residues have been routinely used in over 30 different scFv constructions (Bird *et al.*, 1988; Huston *et al.*, 1988; Whitlow and Filpula, 1991; Whitlow *et al.*, 1993, 1994). The most frequently used linker is that of 15 residues $(Gly_4Ser)_3$ introduced by Huston *et al.* (1988) with the serine residue enhancing the hydrophilicity of the peptide backbone to allow hydrogen bonding to solvent molecules and the glycyl residues to provide the linker with flexibility to adopt a range conformations (Argos, 1990). These properties also prevent interaction of the linker peptide with the hydrophobic interface of the individual domains. Whitlow *et al.* (1993) suggested that scFvs with linkers longer than 15 residues showed higher affinities. In addition, linkers based on natural linker peptides such as the 28-residue interdomain peptide of *Trichoderma reesi* cellobiohydrolase I have been used to link the V_H and V_L domains (Takkinen *et al.*, 1991).

An scFv fragment of antibody NC10 which recognizes a dominant epitope of N9 neuraminidase, a surface glycoprotein of influenza virus, has been constructed and expressed in *E.coli* (Malby *et al.*, 1993). In this scFv, the V_H and V_L domains were linked with a classical 15-residue linker, $(Gly_4Ser)_3$ and the construct contained a hydrophilic octapeptide (FLAG) attached to the C-terminus of the V_L chain as a label for identification and affinity purification (Hopp *et al.*, 1988). The scFv-15 was isolated as a monomer which formed relatively

Table I. DNA sequences of synthetic oligonucleotide duplexes encoding peptide linkers of different lengths inserted into the *Bst*EII and *Sac*I restriction sites of pPOW–scFv NC10 (between the carboxyl of the V_H and the amino terminal of V_L)

Construct	Complementary oligonucleotide pair
scFv-15	5′ GTC ACC GTC TCC GGT GGT GGT GGT TCG GGT GGT GGT GGT TCG GGT GGT GGT GGT TCG GAT ATC GAG CT 3′
	3′ G CAG AGG CCA CCA CCA CCA AGC CCA CCA CCA CCA CCA ACG CCA CCA CCA CCA ACG CTA TAG C 5′
scFv-10	5′ GTC ACC GTC TCC GGT GGT GGT GGT TCG GGT GGT GGT GGT TCG GAT ATC GAG CT 3′
	3′ G CAG AGG CCA CCA CCA CCA ACG CCA CCA CCA CCA ACG CTA TAG C 5′
scFv-5	5′ GTC ACC GTC TCC GGT GGT GGT TCG GAT ATC GAG CT 3′
	3′ G CAG AGG CCA CCA CCA CCA ACG CTA TAG C 5′
scFv-0	5′ GTC ACC GTC TCC GAT ATC GAG CT 3′
	3′ G CAG AGG CTA TAG C 5′

stable dimers and higher molecular mass multimers on freezing at high protein concentrations. The dimers were active, shown to be bivalent (Kortt *et al.*, 1994) and reacted with soluble N9 neuraminidase tetramers to yield a complex with an M_r of ~600 kDa, consistent with four scFvs dimers cross-linking two neuraminidase molecules. Crystallographic studies on the NC10 scFv-15 monomer–neuraminidase complex showed that there were two scFv–neuraminidase complexes in the asymmetric unit and that the C-terminal ends of two V_H domains of the scFv molecules were in close contact (Kortt *et al.*, 1994). This packing indicated that V_H and V_L domains could be joined with shorter linkers to form stable dimeric structures with domains pairing from different molecules and thus provide a mechanism for the construction of bispecific molecules (Hudson *et al.*, 1994, 1995).

In this paper we describe the construction, expression, purification and solution properties of NC10 scFvs with linkers of 0, 5 and 10 residues. The interactions of the NC10 scFvs with tern N9 neuraminidase (Downie *et al.*, 1977; Colman, 1989) and an anti-idiotype antibody (3-2G12) (Metzger and Webster, 1990) which recognizes a private combining site idiotope unique to the parent anti-neuraminidase antibody NC10 were measured in the BIAcore. Our results are compared with other scFv molecules produced with linkers shorter than 12 residues. Holliger *et al.* (1993) constructed scFvs with 0-, 5- and 10-residue linkers, termed diabodies, and showed that these linkers permitted only pairing of domains from different molecules to produce functional bivalent and bispecific dimers. Whitlow *et al.* (1994) also reported the production of a dimeric bispecific scFv using a 12-residue linker. Desplancq *et al.* (1994) constructed scFvs with linkers of 0, 5 and 10 residues and Alfthan *et al.* (1995) constructed scFvs with linkers of 2, 6 and 11 residues and both groups concluded that these scFvs predominantly formed dimers and higher molecular mass multimers. The tertiary structure of a dimeric five-residue linked scFv has been reported (Perisic *et al.*, 1994) but to date there has been no structural data described on the multimeric nature of other scFvs or diabodies with linkers of less than five residues in length.

Materials and methods

Construction of NC10 scFv with 0-, 5- and 10-residue linkers

The NC10 scFv antibody gene construct with a 15-residue linker (Malby *et al.*, 1993) was used for the shorter linker constructions. The NC10 scFv-15 gene was digested successively with *Bst*EII (New England Laboratories) and *Sac*I (Pharmacia) and the polypeptide linker sequence released. The remaining plasmid which contained NC10 scFv DNA fragments was purified on an agarose gel and the DNA concentrated by precipitation with ethanol. Synthetic oligonucleotides (Table I) were phosphorylated at the 5′ termini by incubation at 37°C for 30 min with 0.5 units of T4 polynucleotide kinase (Pharmacia) and 1 mM ATP in One-Phor-All buffer (Pharmacia). Pairs of complementary phosphorylated oligonucleotide primers (Table I) were premixed in equimolar ratios to form DNA duplexes which encoded single-chain linkers of altered lengths. These duplexes were ligated into *Bst*EII–*Sac*I restricted pPOW NC10 scFv plasmid using an Amersham ligation kit. The ligation mixture was purified by phenol–chloroform extraction and precipitated with ethanol in the usual manner and transformed into *E.coli* DH5α (*sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1) and LE392 (*sup*E44, *sup*F58, *hsd*R14, *lac*Y1, *gal*K2, *gal*T22, *met*B1, *trp*R55). Recombinant clones were identified by PCR screening with oligonucleotides directed to the pelB leader and FLAG sequences of the pPOW vector. The DNA sequence of the shortened linker regions were verified by sequencing double-stranded DNA using Sequenase 2.0 (USB).

Expression and purification of the scFvs

The pPOW NC10 scFv constructs with 0-, 5- and 10-residue linkers were expressed as described by Malby *et al.* (1993) for the parent scFv-15. The protein was located in the periplasm as insoluble protein aggregates associated with the bacterial membrane fraction, as found for the NC10 scFv-15 (Kortt *et al.*, 1994). Expressed NC10 scFvs were solubilized in 6 M guanidine hydrochloride, 0.1 M Tris–HCl, pH 8.0, dialysed against PBS, pH 7.4, and the insoluble material was removed by centrifugation. The soluble fraction was concentrated approximately 10-fold by ultrafiltration (Amicon stirred cell, YM10 membrane) as described previously (Kortt *et al.*, 1994) and the concentrate was applied to a Sephadex G-100 column (2.5×60 cm) equilibrated with PBS, pH 7.4; fractions which contained protein were analysed by SDS–PAGE and the scFv was located by Western blot analysis using anti-FLAG M2 antibody (IBI, New Haven, CT). The scFv-containing fractions were pooled, concentrated and purified to homogeneity by affinity chromatography using an anti-FLAG M2 antibody affinity resin (Brizzard *et al.*, 1994). The affinity resin was equilibrated in PBS, pH 7.4, and bound protein was eluted with 0.1 M glycine buffer, pH 3.0, and immediately neutralized with 1 M Tris solution. Purified scFvs were concentrated to ~1–2 mg/ml, dialysed against PBS, pH 7.4, which contained 0.02% (w/v) sodium azide and stored frozen.

The purity of the scFvs was monitored by SDS–PAGE and Western blot analysis as described previously (Kortt *et al.*, 1994). The concentrations of the scFv fragments were determined spectrophotometrically using the values for the extinction coefficient ($\varepsilon^{0.1\%}$) at 280 nm of 1.69 for scFv-15, 1.71 for

scFv-10, 1.73 for scFv-5 and 1.75 for scFv-0 calculated from the protein sequence as described by Gill and von Hippel (1989).

For N-terminal sequence analysis of the intact scFv-0 and scFv-5 and the two lower molecular mass cleavage products, the protein bands obtained on SDS–PAGE were blotted on to a Selex 20 (Schleicher and Schuell) membrane, excised and sequenced using an Applied Biosystems Model 470A gas-phase sequencer.

Preparation of tern N9 neuraminidase and anti-idiotype 3-2G12 Fab'

Tern N9 neuraminidase was isolated from influenza virus following treatment of the virus with pronase and purified by gel filtration as described previously (McKimm-Breschkin *et al.*, 1991).

Monoclonal anti-idiotype antibody, 3-2G12, was prepared in CAF1 mice against NC10 anti-neuraminidase BALB/c monoclonal antibody (Metzger and Webster, 1990). The anti-idiotype 3-2G12 (an IgG1) was isolated from ascites fluid by protein A–Sepharose chromatography (Pharmacia Biotech). Purified antibody was dialysed against 0.05 M Tris–HCl, 3 mM EDTA, pH 7.0, and digested with papain to yield $F(ab')_2$ as described (Gruen *et al.*, 1993). The $F(ab')_2$ was separated from Fc and undigested IgG by chromatography on protein A–Sepharose and pure $F(ab')_2$ was reduced with 0.01 M mercaptoethylamine for 1 h at 37°C and the reaction quenched with iodoacetic acid. The Fab' was separated from the reagents and unreduced $F(ab')_2$ on gel filtration on a Superdex 75 column (HR 10/30) in PBS, pH 7.4.

Size-exclusion FPLC and molecular mass determination

The molecular size and aggregation state of the affinity-purified scFvs were assessed by size-exclusion FPLC on Superose 6 or 12 or Superdex 75 HR 10/30 (Pharmacia) columns in PBS, pH 7.4. The ability of the scFv-0, scFv-5 and scFv-10 to bind to tern N9 neuraminidase and anti-idiotype 3-2G12 Fab' and the size of the complexes formed was also assessed by size-exclusion FPLC on Superose 6 in PBS, pH 7.4. The columns were equilibrated with a set of standard proteins as described previously (Kortt *et al.*, 1994).

The molecular mass of scFv-0, scFv-5 and scFv-10 and that of the complexes formed with tern N9 neuraminidase and anti-idiotype 3-2G12 Fab' with each scFv was determined in 0.05 M phosphate, 0.15 M NaCl, pH 7.4, by sedimentation equilibrium in a Beckman Model XLA ultracentrifuge. A partial specific volume of 0.71 ml/g was calculated for scFv-5 and scFv-0 from their amino acid compositions and a partial specific volume of 0.7 ml/g was calculated for the scFv–neuraminidase complexes from the amino acid compositions of scFvs and the amino acid and carbohydrate (Ward *et al.*, 1983) compositions of neuraminidase. A partial specific volume of 0.73 ml/g was assumed for the scFv-anti-idiotype 3-2G12 Fab' complex. The complexes for ultracentrifugation were prepared by size-exclusion FPLC on Superose 6.

Biosensor binding analysis

The BIAcore biosensor (Pharmacia Biosensor, Uppsala, Sweden), which uses surface plasmon resonance detection and permits real-time interaction analysis of two interacting species (Karlsson *et al.*, 1991; Jonsson *et al.*, 1993), was used to measure the binding kinetics of the different NC10 scFvs. In one series of experiments tern N9 neuraminidase was immobilized on a CM5 sensor chip in 10 mM sodium acetate buffer, pH 4.0, via amine groups using the Amine Coupling Kit (Pharmacia Biosensor) as described previously (Gruen *et al.*, 1993). In a second series of experiments anti-idiotype 3-2G12 Fab' and the NC10 scFv-15 monomer, scFv-10, scFv-5 and scFv-0 were also immobilized at pH 4.0 via their amine groups. Binding analyses were performed in HBS buffer (10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4) at a constant flow rate of 5 µl/min. Neuraminidase, a homotetramer of 190 kDa, was not stable to acid conditions and the surface was regenerated by running the dissociation reaction to completion (~12 000 s) before starting a new binding experiment; this procedure was also used for the immobilized scFvs. Immobilized anti-idiotype (3-2G12) Fab' was regenerated with 10 mM sodium acetate, pH 3.0, with negligible loss of binding activity. Samples for binding analyses were prepared for each experiment by gel filtration on Superdex 75 or Superose 12 to remove any cleavage products or higher molecular mass aggregates which may have formed on storage. The kinetic constants, k_a and k_d, were evaluated using the BIAevaluation 2.1 software supplied by the manufacturer, for binding data where the experimental design correlated with the simple 1:1 interaction model used for the analysis of BIAcore binding data (Karlsson *et al.*, 1994).

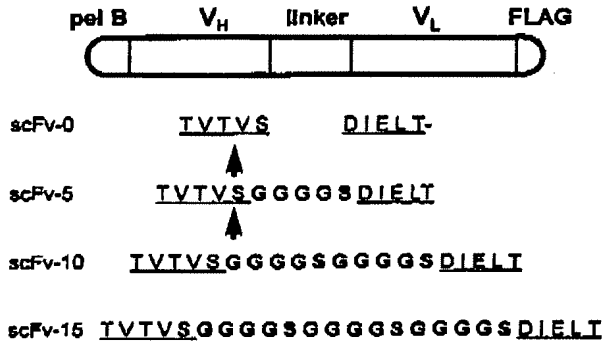

Fig. 1. Schematic diagram of the scFv expression unit showing the sequences of the C-terminus of the V_H domain (residues underlined), N-terminus of the V_L domain (residues underlined) and of the linker peptide (in bold) used in each of the NC10 scFv constructs. The sites of proteolytic cleavage of the scFv-5 and scFv-0 molecules, demonstrated by direct sequencing, are indicated by the arrows.

Results

NC10 scFvs with shorter linkers

The NC10 scFv gene construct (Malby *et al.*, 1993), in which the V_H and V_L domains were linked with the classical 15-residue linker of Huston *et al.* (1988, 1991), was used as a template to construct three new NC10 scFvs with linkers of 10 $(Gly_4Ser)_2$, five (Gly_4Ser) and zero residues as shown in Figure 1. DNA sequencing of the new constructs confirmed that there were no mutations and that the V_H and V_L domains were joined by the designed shorter linker lengths. These constructs, which are referred to as NC10 scFv-10, scFv-5 and scFv-0, where the number refers to the number of residues in the linker, were expressed in *E.coli* using the secretion vector pPOW (Power *et al.*, 1992). Analogously to the parent scFv-15, the majority of the expressed scFv-10, scFv-5 and scFv-0 was located in the periplasm as insoluble protein aggregate and could be partially solubilized by extraction with 6 M guanidine hydrochloride (Malby *et al.*, 1993; Kortt *et al.*,

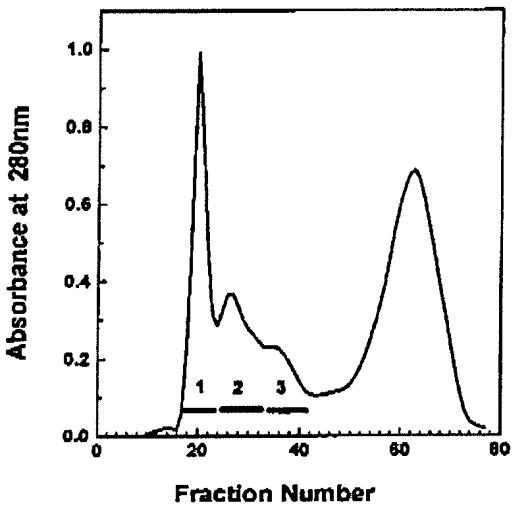

Fig. 2. Sephadex G-100 gel filtration of solubilized NC10 scFv obtained by extraction of the insoluble protein aggregates with 6 M guanidine hydrochloride. A typical elution profile for the scFv-0 is illustrated. The column (2.5×60 cm) was equilibrated with PBS, pH 7.4, and run at a flow rate of 40 ml/h; 10 ml fractions were collected. Aliquots were taken across peaks 1–3 for SDS–PAGE analysis to locate the scFv using protein stain (Coomassie) and Western blot analysis (see Figure 3). The peaks were pooled as indicated by the bars.

Fig. 3. SDS–PAGE analysis of fractions from the Sephadex G-100 gel filtration of scFv-0 shown in Figure 2. Fractions analysed from peaks 1–3 are indicted. (a) Gel stained with Coomassie brilliant blue G-250; (b) Western blot analysis of the same fractions using anti-FLAG M2 antibody.

1994). For each scFv preparation, dialysis was used to remove the guanidine hydrochloride and resulted in both soluble and insoluble scFv.

scFv purification and protein characterization

Soluble scFv-10, scFv-5 and scFv-0 fragments were each purified using a two-step procedure involving gel filtration and affinity chromatography after extraction of the *E.coli* membrane fraction with 6 M guanidine hydrochloride and dialysis to remove denaturant. The solubilized protein obtained was first chromatographed by Sephadex G-100 gel filtration to resolve three peaks (1–3, Figure 2) from a broad, low molecular mass peak. SDS–PAGE and Western blot analysis of fractions across peaks 1–3 showed the presence of scFv in peaks 1 and 2 (fractions 19–30, Figure 3) with most of the scFv in peak 2. In contrast, the previous expression of NC10 scFv-15 resulted in most of the scFv-15 recovered from peak 3 as a monomer (Kortt *et al.*, 1994). Affinity chromatography of peak 2

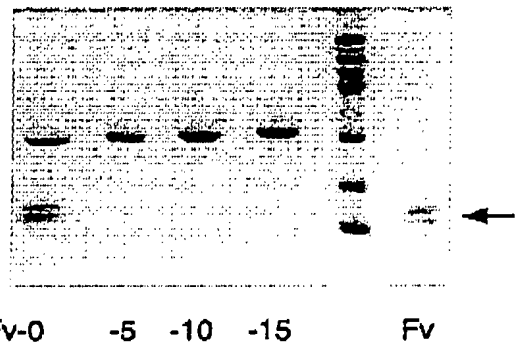

Fig. 4. SDS–PAGE of affinity-purified NC10 scFvs. ScFv-0 shows two lower molecular mass bands of ~14 and 15 kDa (arrowed) corresponding to the V_H and V_L domains produced by proteolytic cleavage of the scFvs during isolation as described in the text. The far right lane shows the monomer peak (Fv) isolated from the scFv-0 preparation (left lane) by gel filtration.

(Figure 2) on an anti-FLAG M2 Sepharose column (Brizzard *et al.*, 1994) yielded essentially homogeneous scFv preparations with a major protein band visible at ~27 kDa by SDS–PAGE analysis (Figure 4); the decreasing size of the linker in the scFv-15, -10, -5 and -0 constructs is apparent from the mobility of the protein bands (Figure 4). ScFv-5 and scFv-0 also contained a small component of the protein as a doublet at ~14 and ~15 kDa (Figure 4) of which the 14 kDa band reacted with the anti-FLAG M2 antibody on Western blotting, consistent with proteolysis in the linker region between the V_H and V_L-FLAG domains (see below).

Affinity chromatography of the Sephadex G-100 peak 1 (Figure 2) of scFv-10 and scFv-5 on an anti-FLAG M2 antibody column yielded scFv preparations which were aggregated; attempts to refold or dissociate the aggregates with ethylene glycol (Kortt *et al.*, 1994) were unsuccessful. This material was not only aggregated but was probably misfolded as it showed no binding activity to N9 neuraminidase or the anti-idiotype 3-2G12 Fab'. All subsequent analyses were performed on scFvs isolated from Sephadex G-100 peak 2.

Molecular mass of scFvs

Gel filtration on a calibrated Superdex 75 column of affinity purified scFvs showed that the scFv-10 (Figure 5) and scFv-5 eluted with an apparent molecular mass of 52 kDa (Table II), indicating that both these molecules are non-covalent dimers of the expressed 27 kDa scFv molecules. Although scFv-5 and scFv-10 yielded predominantly dimer, small amounts of higher molecular mass components were observed (Figure 5b), suggesting that trimer or tetramer forms of the scFv-5 and scFv-10 may also be present. These peaks were not characterized further in this study.

Gel filtration of affinity-purified scFv-0 yielded a single major symmetrical peak with an apparent molecular mass of ~70 kDa (Figure 5, Table II). Since gel filtration behaviour depends on the size and shape of the molecule, the molecular masses of scFv-10, scFv-5 and scFv-0 were determined by sedimentation equilibrium (Table II) to obtain more accurate values. The molecular masses of 54 and 52.4 kDa for scFv-10 and scFv-5 , respectively (Table II), confirmed that they were dimers. The molecular mass of 69 kDa determined for the scFv-0 suggested that it was a trimer composed of three

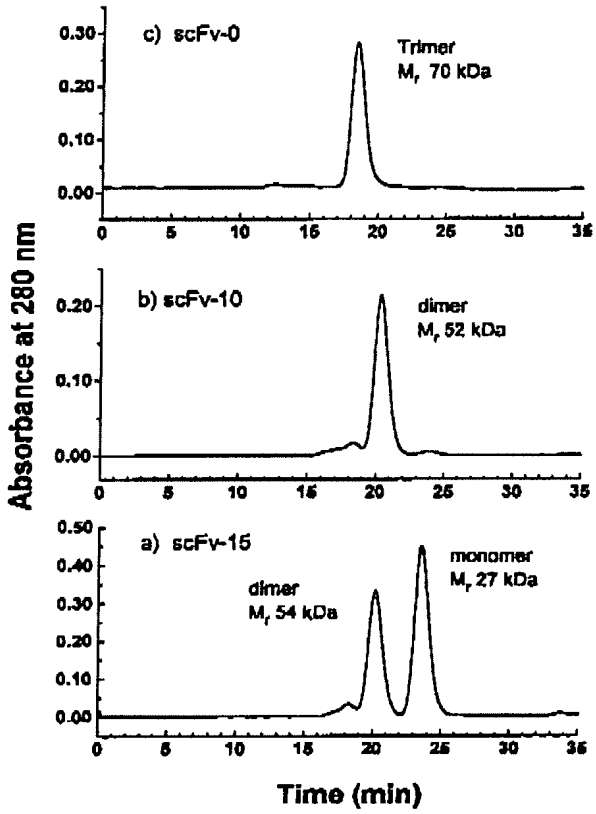

Fig. 5. Size-exclusion FPLC on a calibrated Superdex 75 HR10/30 column (Pharmacia) of affinity-purified NC10 scFvs. The column was equilibrated as described previously (Kortt *et al.*, 1994). (a) Shows the scFv-15 containing monomer, dimer and some higher M_r multimers, (b) shows the scFv-10 with predominantly dimer and (c) shows the scFv-0 eluting as a single peak with an M_r of ~70 kDa. The column was equilibrated with PBS, pH 7.4, and run at a flow rate of 0.5 ml/min.

scFv-0 chains, but this molecular mass is lower than expected for such a trimer (calculated M_r = 78 kDa). Analysis of the sedimentation data gave linear ln*c* versus r^2 plots (Van Holde, 1975) indicating, that under the conditions of the experiment, scFv-5 dimer and scFv-0 trimer showed no dissociation. A detailed analysis of sedimentation behaviour of these molecules over a range of protein concentrations will be reported elsewhere. Furthermore, the sedimentation equilibrium results did not indicate a rapid equilibrium between dimer and trimer species to account for this apparently anomalous molecular mass for scFv-0

Purified scFv-5 and scFv-10 dimers at concentrations of ~1 mg/ml showed no propensity to form higher molecular mass aggregates at 4°C, but on freezing and thawing higher molecular mass multimers were formed (data not shown). These multimers were dissociated readily in the presence of 60% ethylene glycol, which suppresses hydrophobic interactions. In contrast, the scFv-0, on freezing and thawing, showed no propensity to aggregate even at relatively high protein concentrations.

Gel filtration of affinity-purified scFv-5 and scFv-0 showed that some preparations contained a peak eluting with a molecular mass of ~27 kDa; this peak accounted for ~20% of the protein in some samples of scFv-0 and SDS–PAGE analysis showed that it was composed of the two protein bands of ~14 and ~15 kDa (Figure 4). N-Terminal sequence analysis of the two bands from the 27 kDa monomer peak of scFv-5, after SDS–PAGE and blotting on to Selex 20, showed that the 15 kDa band had the sequence of the N-terminus of the V_H domain (QVQLQQSGAE) and the 14 kDa band, which reacted with the anti-FLAG M2 antibody, had the sequence SGGGGSDIEL, indicating that cleavage had occurred at the C-terminal peptide bond of the V_H domain sequence at a V–S bond and not in the linker itself (Figure 1). This material was therefore a proteolytically derived Fv fragment. Minor cleavage also occurred on the N-terminal side of the valine cleaving the T–V bond in the C-terminal TVS sequence of the V_H domain.

N-Terminal analysis of the two bands from the Fv fragment produced during the isolation of the scFv-0 also confirmed that the 15 kDa band was the V_H domain and the 14 kDa band had the N-terminal sequence of VSDIELTQTT which indicated cleavage at the penultimate bond (T–V) in the C-terminal sequence of the V_H domain (Figure 1).

Activity and stability of proteolytically produced Fv

NC10 Fv produced by proteolytic cleavage during preparation of scFv-5 and scFv-0 was shown by gel filtration to be fully active, as estimated by its ability to complex with N9 neuraminidase (data not shown). Gel filtration of a Fv sample at a concentration of 0.3 mg/ml showed no evidence of dissociation into V_H and V_L domains as noted for other Fvs (Glockshuber *et al.*, 1990; King *et al.*, 1993). Furthermore, complete neuraminidase binding activity of the Fv was retained after unfolding in 6 M guanidine hydrochloride at pH 8.0 followed by dialysis against PBS to refold the molecule.

Complexes formed between scFvs (10, 5 and 0) and N9 neuraminidase

Influenza virus neuraminidase, a surface glycoprotein, is a tetrameric protein composed of four identical subunits attached via a polypeptide stalk to a lipid and matrix protein shell on the viral surface (Colman, 1989). Intact and active neuraminidase heads (M_r 190 kDa) are released from the viral surface by proteolytic cleavage in the stalk region (Laver, 1978). The four subunits in the neuraminidase tetramer are arranged such that the enzyme active site and the epitope recognized by NC10 antibody are all located on the upper surface of the molecule (distal from the viral surface). This structural topology permits the binding in the same plane of four scFv-15 monomers or four Fab fragments (Colman *et al.*, 1987; Tulip *et al.*, 1992) such that the tetrameric complex resembles a flattened box or inverted table with the neuraminidase as the top and the four Fab fragments projecting as the legs from the plane at an angle of 45°. This suggests that a bivalent molecule may be able to cross-link two neuraminidase tetramers to form a 'sandwich'-type complex (Tulloch *et al.*, 1986).

Size-exclusion FPLC on a calibrated Superose 6 column showed that both the scFv-10 (Figure 7) and scFv-5 dimers formed stable complexes with soluble neuraminidase with apparent molecular masses of ~600 kDa. The more accurate molecular mass determined by sedimentation equilibrium analysis for the scFv-10 and scFv-5-neuraminidase complexes was 596 kDa. This complex M_r is consistent with four scFv dimers (each 52 kDa) cross-linking two neuraminidase

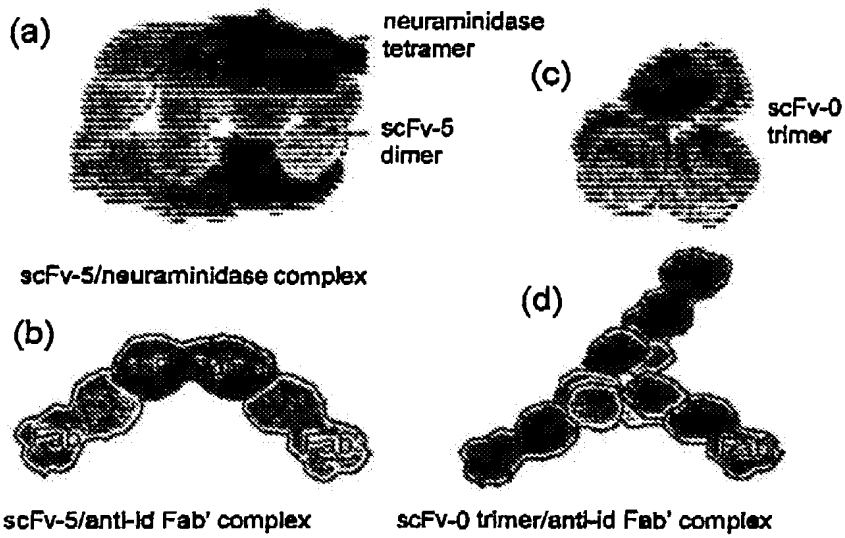

Fig. 6. Sketches illustrating (a) the 'sandwich' complex between two tetrameric neuraminidases and four scFv dimers based on crystallographic data of the neuraminidase–Fab (Tulip *et al.*, 1992; Malby *et al.*, 1994) and scFv-15 monomer (Kortt *et al.*, 1994) complexes and (b) the complex between scFv-5 dimer and anti-idiotype 3-2G12 Fab'. The boomerang-shape of the scFv dimer was taken from data obtained on imaging the scFv-5–anti-idiotype Fab' complex in the electron microscope (P.A.Tulloch and L.Lawrence, unpublished data) and also reported by Perisic *et al.* (1994) for an scFv diabody structure; the angle of projection of ~45° from the plane of the neuraminidase surface was taken from data reported for the neuraminidase–Fab structure. (c) Sketch of the proposed scFv-0 trimer and (d) the scFv-0 binding three anti-idiotype Fab' to form a complex of M_r 212 kDa.

molecules (each 190 kDa) in a 'sandwich' complex, as illustrated schematically in Figure 6, and demonstrates that the scFv-10 and scFv-5 dimers are bivalent.

Gel filtration of the isolated 600 kDa scFv-10–neuraminidase complex showed that it was extremely stable to dilution, with only a small amount of free neuraminidase and scFv-10 appearing when complex at a concentration of 2 nM was run on the Superose 6 column (data not shown). The linearity of the ln*c* versus r^2 plots (Van Holde, 1975) of the sedimentation data showed that both complexes were homogeneous with respect to molecular mass and indicated that discrete and stoichiometric complexes were formed. Complex formation with different molecular ratios of scFv to neuraminidase (from 1:4 to 8:1) yielded only the 600 kDa complex. Interestingly, complexes with four scFv dimers binding to 1 neuraminidase (~400 kDa) or aggregated complexes in which more than two neuraminidases were cross-linked were not observed.

The scFv-0 reacted with tern N9 neuraminidase to form a complex which eluted as a symmetrical peak on the Superose 6 column with an apparent molecular mass of <470 kDa suggesting that less than four scFv-0 trimers (each ~70 kDa) bind per neuraminidase tetramer (190 kDa). Sedimentation equilibrium analysis yielded a more accurate molecular mass of 360 kDa indicating that only two scFv-0 bind per neuraminidase tetramer. These results indicate that in the case of the scFv-0 trimer, only one binding site on this trimer is able to interact with a neuraminidase subunit at the one time and the orientations of the other two (presumed) binding sites are such that cross-linking a second neuraminidase molecule is precluded.

Electron micrographs of the scFv-5– and scFv-10–neuraminidase complexes (P.A.Tulloch and L.Lawrence, in preparation) revealed a discrete image of the complex consistent with the 'sandwich' complex geometry of four scFv dimers cross-linking two neuraminidase molecules as indicated by the solution data.

Complexes formed between scFvs (10, 5 and 0) and anti-idiotype 3-2G12 Fab'

Size exclusion FPLC on Superose 6 showed that anti-idiotype 3-2G12 Fab' formed stable complexes with scFv-15 monomer, scFv-5 and scFv-0. Sedimentation equilibrium analyses of the isolated complexes gave molecular masses consistent with the scFv-15 binding one Fab', scFv-5 binding two Fab's and the scFv-0 binding three Fab' molecules (Table II and Figure 8). The linearity of the ln*c* versus r^2 plots (Figure 8) of the sedimentation data showed that the complexes with scFv-15 monomer and scFv-5 dimer were homogeneous and that discrete and stoichiometric complexes were formed. The equilibrium data for the complex with scFv-0 showed a slight curvature (Figure 8) on linear transformation. The fit to the data yielded an average M_r of 212 000, which corresponds closely to the expected M_r for a complex of three Fab' binding per scFv-0 (Table II). The slight curvature of the transformed data may indicate a small degree of dissociation of the complex under the experimental conditions. The result with the scFv-5 confirmed that the dimer is bivalent and that with the scFv-0 provides direct evidence that the scFv with no linker is a trimer with three active antigen binding sites, as illustrated schematically in Figure 6c and d.

Electron micrographs of the scFv-5–anti-idiotype Fab' complex (M_r 156 kDa) showed a boomerang-shaped structure indicating that there is considerable flexibility in the linker region joining the two scFvs in the crossed dimer (P.A.Tulloch and L.Lawrence, unpublished data). Similar flexibility of a different scFv-5 has recently been modelled (Holliger *et al.*, 1996). This boomerang-shaped structure (Figure 6b), rather than a linear structure, can readily accommodate the 45° angle

Table II. Molecular mass of NC10 scFvs and of the complexes formed with tern N9 neuraminidase and anti-idiotype 3-2-G12 Fab' fragment

Construct		Molecular mass	
		Measured	Calculated
scFv-15	Monomer	27 300	27 100
	Dimer	54 300	54 200
scFv-10	Dimer	54 000	53 570
scFv-5	Dimer	52 440	52 940
scFv-0	Trimer	70 000[a]	78 464
		69 130	
		scFv-tern N9 neuraminidase complex	
		Measured	Calculated
scFv-15	Monomer	298 000	298 400
	Dimer	610 000	596 800
scFv-10	Dimer	596 000	594 280
scFv-5	Dimer	595 000	591 760
scFv-0	Trimer	360 000	
		scFv-anti-idiotype 3-2-G12 Fab' complex	
		Measured	Calculated
scFv-15	Monomer	77 900	77 100
scFv-10	Dimer	nd	
scFv-5	Dimer	156 000	152 940
scFv-0	Trimer	212 000[b]	220 000

Molecular mass determined in 0.05 M phosphate, 0.15 M NaCl, pH 7.4, by sedimentation equilibrium analysis in a Beckman Model XLA ultracentrifuge. The molecular masses of the complexes were calculated using an M_r of 50 000 for the Fab' and 190 000 for tern N9 neuraminidase.
[a]Molecular mass estimated by gel filtration on Superdex 75 in 0.05 M phosphate, 0.15 M NaCl, pH 7.4, at a flow rate of 0.5 ml/min at 20°C.
[b]Apparent average molecular mass obtained by fitting data in Figure 8 assuming a single species.

of projection of the scFv from the plane of the neuraminidase required for four dimers to cross-link simultaneously two neuraminidase molecules in the 'sandwich' complex as indicated in Figure 6a.

Binding interactions of NC10 scFvs measured on the BIAcore

Binding of scFvs to tern N9 neuraminidase.
Sensorgrams for the interaction of scFv-15 monomer and scFv-10 dimer with immobilized N9 neuraminidase are shown in Figure 9. The scFv-15 monomer–neuraminidase complex dissociates relatively rapidly so that complete dissociation was obtained in about 6000 s. Sequential binding experiments were performed by allowing complete dissociation with continuous buffer flow since the binding activity of immobilized neuraminidase was irreversibly destroyed by acidic regeneration conditions. Although unstable to acidic conditions tern N9 neuraminidase was found to be stable to prolonged exposure (>2 weeks) to the HBS buffer at 25°C when immobilized on the sensor surface.

The apparent association and dissociation rate constants were determined for the binding of scFv-15 monomer to immobilized neuraminidase (Table III). In the analysis it was assumed that the univalent scFv-15 monomer interacted with the epitope on each neuraminidase subunit in an independent, non-cooperative manner and the data were analysed according to a 1:1 interaction model of the type A + B ⇌ AB, using non-linear regression.

In contrast, scFv-10, a bivalent dimer, formed a complex with tetravalent neuraminidase which dissociated more slowly (Figure 9). This slower dissociation can be attributed to an avidity effect of a bivalent molecule binding to tetrameric neuraminidase and kinetic analysis was not performed for this interaction. The slower dissociation rate requires that the surface be regenerated to dissociate the complex formed and, as this was not possible with immobilized neuraminidase, sequential binding experiments could not be performed with the dimeric scFvs.

Binding of scFvs to anti-idiotype 3-2G12 Fab'.
Immobilized 3-2G12 Fab' could be regenerated with 10 µl of 0.01 M sodium acetate buffer, pH 3.0, without loss of binding activity. A comparison of the binding of the scFv-15 monomer, scFv-10 and scFv-5 dimers and scFv-0 trimer (Figure 10) showed the same behaviour as observed with immobilized neuraminidase; the monomer dissociated rapidly and non-linear least-squares analysis of the dissociation and association phase, using the single exponential form of the rate equation, gave a good fit to the experimental data. The rate constants determined are given in Table III. The scFv-10 and scFv-5 dimers and scFv-0 trimer–anti-idiotype complexes showed apparently slower dissociation (Figure 10) consistent with multivalent binding as discussed above and kinetic analysis was not performed because this effect invalidates the 1:1 interaction model used to evaluate BIAcore data. To resolve this problem, the interaction format was inverted by immobilization of each scFv and using the anti-idiotype Fab' as the analyte. This achieves experimentally a 1:1 interaction model required to determine the rate constants.

Binding of anti-idiotype 3-2G12 Fab' to immobilized scFv-15 monomer and scFv-10, scFv-5 and scFv-0.

(i) scFv-15 monomer.
Although the scFv-15 monomer was readily immobilized (~2000 RU), less than 10% of the protein was active as indicated by the total amount of anti-idiotype Fab' that could be bound to the surface as calculated from the RU increase. Logarithmic transformation of the dissociation phase data showed significant deviation from linearity which permitted only approximate values of the binding constants to be estimated (Table III).

(ii) scFv-10, scFv-5 and scFv-0.
In contrast, the three scFvs with the shorter linkers were not readily immobilized via their amine groups since only 200–550 RU of protein could be immobilized after several injections of protein at a flow rate of 2 µl/min. Binding experiments with anti-idiotype 3-2G12 Fab' indicated that ~30–50% of the immobilized scFv-10, scFv-5 and scFv-0 were active as calculated from the total bound RU response. As for immobilized scFv-15 monomer analysis of the data showed deviation from linearity on logarithmic transformation of dissociation data and poor fits when the data were analysed by non-linear regression. These non-ideal effects associated with BIAcore binding data may arise either from the rate of molecular diffusion to the surface contributing to the kinetic constants (mass transfer effect) (Glaser, 1993; Karlsson *et al.*, 1994) or binding heterogeneity of the immobilized molecules resulting from the non-specific immobilization procedure used (Kortt *et al.*, 1997) or both. These effects contribute to lowering the measured rate constants and affect the estimated binding

Table III. Apparent kinetic constants for the binding of NC10 scFvs to immobilized tern N9 neuraminidase and anti-idiotype 3-2-G12 Fab' fragment determined in the BIAcore

Immobilized ligand	Analyte	Apparent k_a (M^{-1} s^{-1})	Apparent k_d (s^{-1})	Apparent K_a (M^{-1})
Neuraminidase	scFv-15 monomer	$2.6 \pm 0.3\times10^{5}$	$5.2 \pm 0.3\times10^{-3}$	$5.0 \pm 0.9\times10^{7}$
3-2-G12 Fab'	scFv-15 monomer	$7.4 \pm 0.6\times10^{5}$	$1.74 \pm 0.06\times10^{-3}$	$4.2 \pm 0.5\times10^{8}$
scFv-15 monomer	3-2-G12 Fab'	$5 \pm 1\times10^{5}$	$2.1 \pm 0.1\times10^{-3}$	$2.5 \pm 0.63\times10^{8}$
scFv-10 dimer	3-2-G12 Fab'	$3.7 \pm 0.4\times10^{5}$	$2.9 \pm 0.2\times10^{-3}$	$1.3 \pm 0.23\times10^{8}$
scFv-5 dimer	3-2-G12 Fab'	$3.5 \pm 0.9\times10^{5}$	$3.3 \pm 0.1\times10^{-3}$	$1.06 \pm 0.3\times10^{8}$
scFv-0 trimer	3-2-G12 Fab'	$2.6 \pm 0.1\times10^{5}$	$2.3 \pm 0.1\times10^{-3}$	$1.13 \pm 0.9\times10^{8}$

The kinetic constants were evaluated from the association and dissociation phase using non-linear fitting procedures described in BIAevaluation 2.1. The binding experiments were performed in 10 mM HEPES, 0.15 NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4, at a flow rate of 5 µl/min. Tern N9 neuraminidase (1360 RU), 3-2-G12 Fab' (1000 RU), NC10 scFv-15 monomer (2000 RU) and NC10 scFv-10 dimer (200 RU), scFv-5 dimer (200 RU) and scFv-0 trimer (450 RU) were immobilized via amine groups using the standard NHS–EDC coupling procedure.

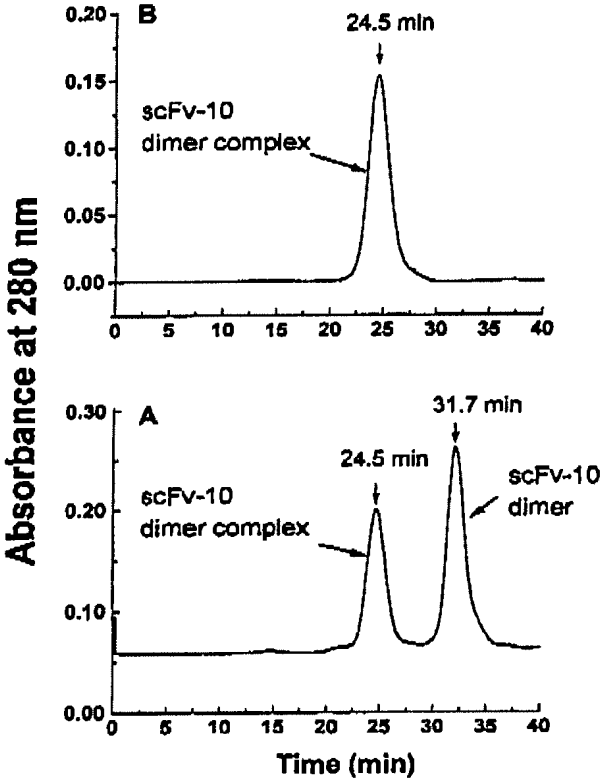

Fig. 7. Size-exclusion FPLC on a calibrated Superose 6 HR10/30 column (Pharmacia) of the complex formed on the interaction of scFv-10 and tern N9 neuraminidase. (a) Shows the complex formed in the presence of an excess of scFv-10 and (b) shows the rechromatography of the isolated complex peak from (a). Unbound neuraminidase elutes at 29 min. The column was equilibrated with PBS, pH 7.4, and run at a flow rate of 0.5 ml/min.

constants (Table III). A comparison of the rate constants for the four scFvs with the different length linkers shows that the apparent affinity of each scFv is similar. Increases in affinity that are reported for dimeric scFvs therefore arise from an avidity effect when dimers are used as analytes in either BIAcore biosensor or ELISA affinity measurements.

Discussion

Design of scFv with shorter linkers

The design of linker length in this study was initially based on the precise distances between N- and C-terminal residues from the crystal structure of NC10 scFv-15 (Kortt *et al.*, 1994). In previous studies, the design of flexible linker peptides to join V_H and V_L domains to produce scFvs (Huston *et al.*, 1991; Ragg and Whitlow, 1995) and the effect of the linker structure on the solution properties of scFvs has been investigated (Holliger *et al.*, 1993; Desplancq *et al.*, 1994; Whitlow *et al.*, 1994; Alfthan *et al.*, 1995; Solar and Gershoni, 1995). ScFvs with the classical 15-residue linker, $(Gly_4Ser)_3$, described by Huston *et al.* (1989, 1991) can exist as monomers, dimers and higher molecular mass multimers (Holliger *et al.*, 1993; Kortt *et al.*, 1994; Whitlow *et al.*, 1994). This propensity of scFvs to dimerize was exploited further by Whitlow *et al.* (1994) to make bispecific dimers by linking V_H and V_L domains of two different antibodies (4-4-20 and CC49) to form a mixed scFv and then forming an active heterodimer by refolding a mixture of the two scFv in the presence of 20% ethanol, 0.5 M guanidine hydrochloride. The main disadvantage of this approach with 15-residue or longer linkers is that different V_H and V_L pairings show different dimerization and dissociation rates.

Linkers of less than 12 residues are too short to permit pairing between V_H and V_L domains on the same chain and have been used to force an intermolecular pairing of domains with the complementary domains of another chain to yield a 'crossed dimer' with two antigen binding sites, termed diabodies (Holliger *et al.*, 1993; Hudson *et al.*, 1995). ScFvs with shorter linkers ranging from 11 to zero residues have been described but in most studies detailed molecular mass analyses have been lacking. Holliger *et al.* (1993) made constructs with five- and zero-residue linkers and reported both scFvs to be dimers. Desplancq *et al.* (1994) described a series of scFvs with linkers of 10, 5 and 0 residues and showed by FPLC analysis that these scFvs were predominantly dimers with minor amounts of monomer. The possibility that the monomer contained a proteolytically produced Fv was not addressed. Solar and Gershoni (1995) found that the scFv 4-4-20/9 with a nine-residue linker was susceptible to proteolysis but the size was not characterized. Alfthan *et al.* (1995) showed that a series of anti-2-phenyloxazolone scFvs with linkers of 11, 6 and 2 residues had a tendency to form multimers but the molecular masses of the different constructs were not determined.

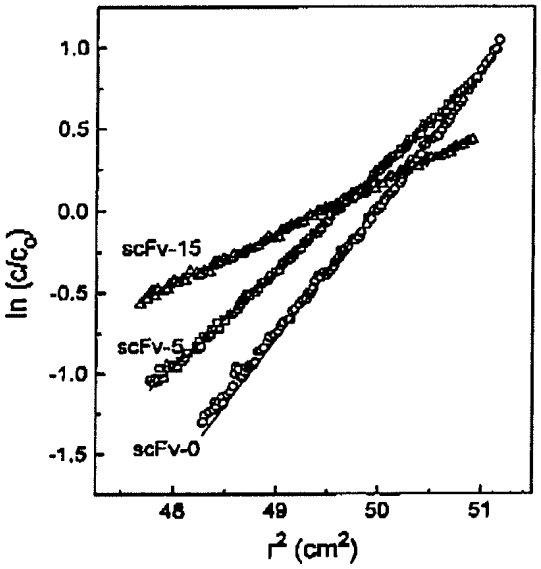

Fig. 8. Sedimentation equilibrium data for complexes of anti-idiotype 3-2G12 Fab' and NC10 scFv-15 monomer, scFv-5 dimer and scFv-0 trimer. The complexes were isolated by size-exclusion chromatography on Superose 6 in 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.4. Experiments were conducted at 1960 *g* at 20°C for 24 h using double-sector centrepiece and 100 µl of sample. The absorbance at 214 nm was determined as a function of radius in cm. Data for the complexes of anti-idiotype 3-2G12 Fab' with scFv-15 monomer (Δ), scFv-5 (□) and scFv-0 (○) are shown.

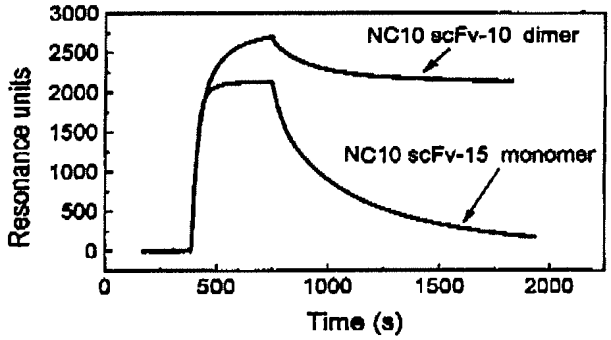

Fig. 9. Sensorgrams showing the binding of NC10 scFv-15 monomer and NC10 scFv-10 dimer at a concentration of 10 µg/ml to immobilized tern N9 neuraminidase (1360 RU immobilized). An injection volume of 30 µl and a flow rate of 5 µl/min were used. For the NC10 scFv-15 monomer the baseline response returned to the starting value after 6000 s and NC10 scFv-15 monomer at a series of concentrations (15–231 nM) was run to measure the rate constants for this interaction (see Table II).

An important aspect in the design of scFvs with a reduced linker length is the precise definition of the N- and C-terminal residues of V_H and V_L to be linked. These residues should be constrained in the structure by having H-bond contacts to other framework V-domain residues or otherwise will form part of the flexible linker. For NC10 we could define precisely the constrained residues using the structural data from the X-ray diffraction analysis of the neuraminidase–Fab and neuraminidase–scFv complexes, including the binding interfaces (Kortt

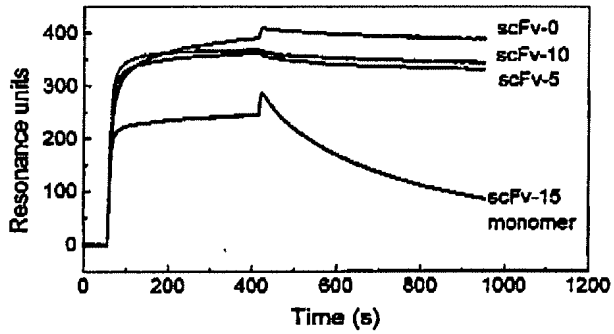

Fig. 10. Sensorgrams showing the binding of NC10 scFv-15 monomer, scFv-10 dimer, scFv-5 dimer and scFv-0 trimer each at a concentration of 10 µg/ml to immobilized anti-idiotype 3-2G12 Fab' (1000 RU). An injection volume of 30 µl and a flow rate of 5 µl/min were used. The surface was regenerated with 10 µl of 10 mM sodium acetate, pH 3.0, after each binding experiment.

et al., 1994; Malby *et al.*, 1994). These structures had been refined to nominal resolutions of 2.4 and 3.0 Å, respectively. The C-terminal residues of NC10 V_H were defined as TVS (residues 110–112), where Thr110 and Val111 contacted framework residues Val12 and Ser87 respectively. The C-terminal residue was defined as Ser112 from the electron density map. This residue was well ordered and had limited flexibility but was not in direct H-bonded contact to other V_H-domain residues. The N-terminal residues of NC10 V_L were defined as DIE, in which side chain atoms in Asp1 and Ile2 contact framework residues Glu3 and Thr97 respectively, whilst regular β-sheet H-bonding began with Glu3. From this analysis we reasoned that the reduced linkers should join Ser112 of VH to Asp1 of V_L and the zero linker should be a direct ligation of these residues. Thus the linkers of 10 and five residues reflect the actual linker lengths and do not involve an additional contribution from flexible C-terminal or N-terminal residues

scFv dimers

NC10 scFv with a 15-residue linker has been shown previously to form both monomers and dimers (Kortt *et al.*, 1994) as described for most other scFvs with linkers of this length or longer. In the case of NC10 scFv-15 the formation of the dimer was concentration dependent and the dimer was relatively stable. NC10 scFv-10 and scFv-5 form exclusively dimers of 52 kDa.

The NC10 scFv dimers are bivalent with two antigen binding sites pointing in opposite directions since scFv-10 and scFv-5 can bind either two anti-idiotype 3-2G12 Fab' fragments or form a 'sandwich' complex with neuraminidase consistent with four scFv dimers cross-linking two neuraminidase tetramers (Figure 6). In the crystal structure of NC10 scFv-15 (Kortt *et al.*, 1994), two scFv molecules were observed to be joined back-to-back with a calculated distance of 3.4 nm in a 'conventional' scFv alignment or 1.8 nm in a diabody alignment. The actual linker position could not be determined from the calculated electron density, presumably owing to its flexibility, but we believe the most likely structure is that of two conventional scFv monomers joined back-to-back. It is not possible for scFvs with linkers of less than 12 residues to adopt the monomer conformation without distorting the V-domain framework residues. However, from the calculated distances and assuming each residue spans ~0.34 nm, it is

possible that diabody forms of scFv-10 but not scFv-5 could still adopt this back-to-back configuration. An alternative structure that could be adopted by both scFv-10 and scFv-5 is as 'twisted' diabodies in which only V_H domains are in contact between Fv modules (Perisic *et al.*, 1994). The minimum linker required for 'twisted' diabodies is one or two flexible residues and a structure of this form has been modelled by Holliger *et al.* (1993) and confirmed in a crystal structure of a five-residue diabody (Perisic *et al.*, 1994). As noted by Perisic *et al.* (1994), it was difficult to fit scFv-0 to this structure, even with severe rotations of the V_H domains.

scFv trimer

The NC10 scFv-0 on FPLC and sedimentation equilibrium analysis yielded a molecular mass of 70 kDa, significantly higher than expected for a dimer (52 kDa) and less than that for a trimer (78 kDa). Binding experiments with anti-idiotype 3-2G12 Fab' showed that the scFv-0 formed a complex of M_r of 212 kDa consistent with three Fab' fragments binding per scFv-0. This result confirmed that the 70 kDa scFv-0 was a trimer and that three pairs of V_H and V_L domains interact to form three active antigen combining sites. This scFv-0 structure showed no propensity to form higher molecular mass multimers but the structure in the peptide region joining the V_H and V_L domains is clearly strained as it was significantly more susceptible to proteolysis than the scFv-5. The scFv-0 also bound to neuraminidase but the arrangement of the antigen combining sites is such that a second antigen binding site on NC10 scFv-0 could not cross-link the neuraminidase tetramers into 'sandwiches' as shown for the scFv-10 and scFv-5 dimers. A computer graphic model was constructed for a zero-residue linked scFv trimer, based on the NC10 scFv coordinates, using circular threefold symmetry (Figure 11). Ser 112, the C-terminal residues of V_H domains, were joined by single peptide bonds to Asp1, the N-terminal residues of V_L domains. The V_H and V_L domains were rotated around the peptide bond to minimise steric clashes between domains. The Fv conformation and CDR positions were consistent with the molecule possessing trivalent affinity. The low contact area between Fv modules, across the V_H–linker–V_L interface, might account for the increased proteolytic susceptibility of scFv trimers compared with scFv-5 residue linked dimers. Although the protein chemical data would not differentiate between symmetric or non-symmetric trimers, the model clearly demonstrated that zero-linked scFvs could form trimers without interdomain steric constraints.

Binding affinities of NC10 scFvs

Binding studies using the BIAcore biosensor showed that all NC10 scFvs with the different linker lengths bound to immobilized neuraminidase and anti-idiotype 3-2G12 Fab'. In the case where the dimers and trimer were used as analyte, the kinetic constants were not evaluated because multivalent binding resulted in an avidity effect and invalidated the kinetic interaction model. Experiments with the immobilized scFvs showed that the affinity of each antigen combining site for anti-idiotype 3-2G12 Fab' was essentially identical (Table III) and that increases in affinity that have been reported elsewhere for dimers (and higher molecular mass multimers) are clearly due to an avidity effect. The loss of up to 90% activity on immobilization of the NC10 scFvs and the deviation from ideal behaviour indicate that these reactions are affected by problems of ligand binding heterogeneity introduced by the amine immobilization procedure. The complex formation data

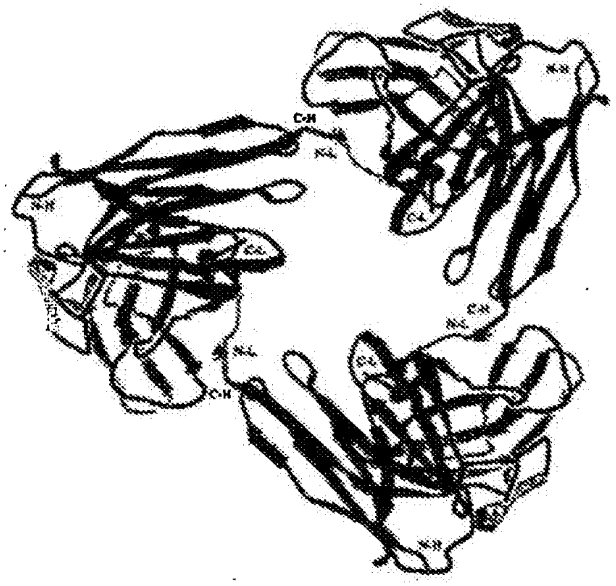

Fig. 11. Model of NC10 scFv-0 trimer constructed with circular threefold symmetry using the O molecular graphics package (Jones *et al.*, 1991) from the coordinates of the NC10 Fv domain in PDB entry 1NMB (Malby *et al.*, 1994). The threefold axis is shown out of the page. The V_H and V_L domains are shaded dark grey and light grey, respectively. CDRs are shown in black and the peptide bonds (zero-residue linkers) joining the carboxy terminus of V_H to the amino terminus of the V_L in each single-chain are shown with a double line. Amino (N) and carboxy (C) termini of the V_H (H) and V_L (L) domains are labelled. MOLSCRIPT (Kraulis, 1991).

in solution supported the conclusion that the scFvs bound to antigen with full stoichiometry.

Conclusion

This study has shown that linkers of 10 or five residues joining the NC10 V_H and V_L domains resulted in the exclusive formation of bivalent dimers. The pairing of V_H and V_L domains from different molecules results in non-covalently crossed diabodies. For the scFv-5 and scFv-10 constructs monomers do not form and any observed monomeric species are proteolytically produced Fv fragments. The direct linkage of NC10 V_H and V_L domains as scFv-0 produced a trimer with three antigen combining sites capable of binding antigen. We believe the NC10 scFv-0 molecule is the first report of a stable trimeric scFv. Previous scFvs with zero linker have been reported to be dimers which suggests that C-terminal and N-terminal residues in those constructs have some flexibility and may act as a short linker (Holliger *et al.*, 1993). Indeed, the allowed flexibility between Fv modules of a five-residue linked diabody has recently been modelled (Holliger *et al.*, 1996) and presumably linkers of less than five residues would severely restrict this flexibility. Studies to determine the minimum linker length required for the NC10 scFv construct to form a trimer are in progress.

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